



REC'D 11 NOV 2003

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Patent application No.: PA 2003 00076

Date of filing: 22 January 2003

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Title: An improved Bret assay

IPC: -

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31 October 2003

  
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**AN IMPROVED BRET ASSAY**

**FIELD OF THE INVENTION**

- 5 The present invention describes an improved BRET assay, wherein the BRET signal is enhanced and/or prolonged. The improved BRET assay comprises the following steps:
- i) adding a substrate to a cell comprising GPCR-Rluc fusion protein and a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is mutated,
  - li) adding a ligand to obtain, if possible, a GPCR-Rluc/ $\beta$ -arrestin-GFP complex, and
  - 10 iii) measuring a BRET signal to obtain a BRET ratio

the improvement leads to an increased BRET ratio compared with the ratios obtained by use of the same process employing a  $\beta$  arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is the wild type  $\beta$ -arrestin or employing a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$ -

15 arrestin is a  $\beta$ -arrestin specifically mutated so that it acts on the receptor independent of the receptors phosphorylation state.

**BACKGROUND OF THE INVENTION**

20 *BRET assay*

BRET (Bioluminescence Resonance Energy Transfer) assay is a protein-protein interaction assay. It is based on energy transfer from a bioluminescent donor to a fluorescent acceptor protein. This technology uses a Renilla luciferase (Rluc) as the donor and a Green Fluorescent Protein (GFP) as the acceptor molecule.

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Rluc emits blue light (e.g. at 400 nm) in presence of its substrate. If a GFP molecule is in close proximity to the Rluc, it absorbs the blue light energy and re-emits green light (e.g. at 515 nm). The BRET signal, therefore, is measured by the amount of green light emitted by GFP as compared to the blue light emitted by Rluc. The ratio of green to blue

30 increases as the two proteins are brought into proximity. BRET assays are performed by genetically fusing Rluc and GFP to biological partners that are expected to interact in a cell-based assay.

However, in certain situations where a GPCR-arrestin based assay is used, the BRET

35 signal is relatively weak and short termed. Thus, there is a need for improving the BRET assay in order to obtain a prolonged and/or enhanced signal.

## DETAILED DISCLOSURE OF THE INVENTION

Accordingly, the present invention provides an improved BRET assay that comprises the following steps:

- 5 i) adding a substrate to a cell comprising GPCR-Rluc fusion protein and a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is mutated,
  - ii) adding a ligand to obtain, if possible, a GPCR-Rluc/ $\beta$ -arrestin-GFP complex, and
  - iii) measuring a BRET signal to obtain a BRET ratio
- 10 the improvement leads to an increased BRET ratio compared with the ratios obtained by use of the same process employing a  $\beta$  arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is the wild type  $\beta$ -arrestin or employing a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is a  $\beta$ -arrestin is specifically mutated so that it acts on the receptor independent of the receptors phosphorylation state.

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### *G protein-coupled receptors for use in the present invention*

- The G protein-coupled receptors (GPCRs) constitute the largest family of proteins in the human genome and function as receivers of all kinds of chemical signals. The spectrum of hormones, neurotransmitters, paracrine mediators etc., which act through G-protein
- 20 coupled receptors includes all kinds of chemical messengers: Ions (calcium ions acting on the parathyroid and kidney chemosensor), amino acids (glutamate and  $\gamma$ -amino butyric acid - GABA), monoamines (catecholamines, acetylcholine, serotonin, etc.), lipid messengers (prostaglandins, thromboxane, anandamide, (endogenous cannabinoid), platelet activating factor, etc.), purines (adenosine and ATP), neuropeptides (tachykinins,
- 25 neuropeptide Y, endogenous opioids, cholecystokinin, vasoactive intestinal polypeptide (VIP), plus many others), peptide hormones (angiotensin, bradykinin, glucagon, calcitonin, parathyroid hormone, etc.), chemokines (interleukin-8, RANTES, MIP-1alpha etc.), glycoprotein hormones (TSH, LH/FSH, choriongonadotropin, etc.), as well as proteases (thrombin). In our sensory systems, G-protein coupled receptors are involved both as the
- 30 light sensing molecules in the eye, i.e. rhodopsin and the color pigment proteins, and as several hundreds of distinct odorant receptors in the olfactory system as well as a large number of taste receptors. Structurally, G protein coupled receptors (GPCRs) are characterized by seven hydrophobic helical transmembrane segments connected by intra- and extracellular loops and are accordingly often referred to as 7TM receptors.

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Examples of 7TM receptors are the receptors for (– in brachet the receptor subtypes are mentioned): acetylcholine (m1-5), adenosine (A1-3) and other purines and purimidines

(P2U and P2Y1-12), adrenalin and noradrenalin ( $\alpha$ 1A-D,  $\alpha$ 2A-D and  $\beta$ 1-3), amylin, adrenomedullin, anaphylatoxin chemotactic factor, angiotensin (AT1A, -1B and -2), apelin, bombesin, bradykinin (1 and 2), C3a, C5a, calcitonin, calcitonin gene related peptide, CD97, conopressin, corticotropin releasing factor (CRF1 and -2), calcium, cannabinoid

5 (CB1 and -2), chemokines (CCR1-11, CXCR1-6, CX3CR and XCR), cholecystokinin (A-B), corticotropin-releasing factor (CRF1-2), dopamine (D1-5), eicosanoids, endothelin (A and B), fMLP, Frizzled (Fz1,2,4,5 and 7-9), GABA (B1 and B2), galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like peptide I and II, glutamate (1-8), glycoprotein hormone (e.g. FSH, LSH, TSH, LH), growth hormone releasing hormone, growth hormone

10 secretagogue / Ghrelin, histamine (H1-4), 5-hydroxytryptamine (5HT1A-1F, -2A-C and -4-7), leukotriene, lysophospholipid (EDG1-4), melanocortins (MC1-5), melanin concentrating hormone (MCH 1 and 2), melatonin (ML1A and 1B), motilin, neuromedin U, neuropeptide FF (NFF1 and 2), neuropeptide Y (NPY1,2,4,5 and 6), neurotensin (1 and 2), nociceptin, odor components, opioids ( $\kappa$ ,  $\delta$ ,  $\mu$  and  $\chi$ ), orexins (OX1 and -2), oxytocin, parathyroid

15 hormone/parathyroid hormone-related peptides, pheromones, platelet-activating factor, prostaglandin (EP1-4 and F2) prostacyclin, pituitary adenylate activating peptide, retinal, secretin, smoothened, somatostatins (SSTR1-5), tachykinins (NK1-3), thrombin and other proteases acting through 7TM receptor, thromboxane, thyrotropin-releasing hormone, vasopressin (V1A, -1B and -2), vasoactive intestinal peptide, urotensin II, and virally

20 encoded receptors (US27, US28, UL33, UL78, ORF74, U12, U51); and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned such as mas proto-oncogene, EBI (I and II), lactophilin, brain specific angiogenesis inhibitor (BAI1-3), EMR1, RDC1 receptor, GPR12 receptor or GPR3 receptor, and 7TM proteins coded for in the human genome but for which no endogenous

25 ligand has yet been assigned.

#### *Arrestins role in receptor signalling*

Arrestins play an important role in the regulation of 7TM receptor responsiveness by terminating the G protein mediated signal. Arrestins are cytosolic proteins, which upon

30 agonist binding to 7TM receptors are translocated to the activated and usually phosphorylated receptor within seconds or minutes after agonist stimulation. Full inactivation of 7TM receptor signaling is achieved through binding of one of a family of arrestin molecules, which sterically hinder G protein binding.

35 Arrestin functions as an adaptor protein, which will connect the receptor to clathrin and AP-2, which results in sequestration of the receptor into intracellular vesicles of the

endosomal pathway in which dynamin plays an important role in the actual vesicular sequestration process.

- The family of arrestins has at least four members showing a high degree of amino acid
- 5 homology and classified primarily on the basis of tissue distribution. They include (i) visual arrestin and (ii) C-arrestin, which are mostly restricted to the eye, and the non-visual-arrestins (iii)  $\beta$ -arrestin1 and (iv)  $\beta$ -arrestin2, distributed ubiquitously in almost every tissue.  $\beta$ -arrestins share more than 70% amino acid identity.
- 10 Arrestins are composed of two structural and functional parts, an amino-terminal domain, which binds to the receptor and a carboxyl-terminal domain, which connects to proteins involved in receptor-sequestration, such as clathrin and AP-2 (adaptor protein 2). Visual arrestins, which mainly interacts with the rhodopsin receptor, are very weak in their clathrin-association and are in general not considered to be capable of mediating receptor
- 15 internalisation.

- As described above arrestin are translocated to the activated and usually phosphorylated GPCR within minutes after agonist stimulation. This interaction is universal for almost all GPCR's upon activation. Thus, a BRET assay based on the GPCR-arrestin interaction,
- 20 wherein GPCR is fused with Rluc and arrestin is fused with GFP, is a very useful assay for a wide range of receptors, and it also provides means for the discovery of ligands that interact with GPCRs of unknown function i.e. orphan GPCRs.

- However, as described above, arrestin functions as an adaptor protein, which will connect
- 25 the receptor to clathrin and AP-2, which results in sequestration of the receptor into intracellular vesicles. After internalization of the receptor/arrestin complex, arrestin will dissociate from the receptor and the BRET signal is terminated. The dissociation kinetics can be fast or slow depending on the receptor type. For Class A type receptors, the dissociation is usually fast, whereas for Class B type receptors the dissociation is slower.

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The present invention describes an improved BRET assay wherein the BRET signal is enhanced and/or prolonged.

- In the present context the term "BRET ratio" is intended to mean the ratio of green light
- 35 emitted by GFP as compared to the blue light emitted by Rluc. In the present context the terms "BRET signal" and "BRET ratio" are intended to have the same meaning.

A "ligand" is intended to include a substance that either inhibits or stimulates the activity of a receptor and/or that competes for the receptor in a binding assay. An "agonist" is defined as a ligand increasing the functional activity of a biological target molecule. An "antagonist" is defined as a ligand decreasing the functional activity of a biological target molecule either by inhibiting the action of an agonist or by its own intrinsic activity. An "inverse agonist" (also termed "negative antagonist") is defined as a ligand decreasing the basal functional activity of a biological target molecule

In the present context the term "improved BRET assay" denotes an assay where the BRET ratio is increased by at least about 5% such as, e.g., at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or more.

In the present context, the term " $\beta$ -arrestin that is specifically mutated so that it acts on the receptor independent of the receptors phosphorylation state" means that  $\beta$ -arrestin is mutated deliberately with the purpose of becoming phosphorylation independent. An example of such a mutation is R169E human  $\beta$ -arrestin-2, wherein arginine has been changed to glutamic acid.  $\beta$ -arrestins being mutated for other purposes, but accidentally also being phosphorylation independent, are not encompassed in this definition.

Since the BRET signal is dependent on the association/dissociation of the GPCR-Rluc/ $\beta$ -arrestin-GFP complex, prevention of the dissociation of the complex will enhance and/or prolong the BRET signal.

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Thus, the invention also provides an improved assay, wherein the separation of  $\beta$ -arrestin-GFP from GPCR-Rluc/ $\beta$ -arrestin-GFP complex is delayed and/or inhibited.

As described above, the GPCR-Rluc/ $\beta$ -arrestin-GFP complex dissociates when the complex is internalized. Thus, inhibition of the internalization will prevent dissociation and accordingly, the BRET signal will be enhanced and/or prolonged. Accordingly, the invention also relates to an improved assay wherein the internalization of GPCR-Rluc/ $\beta$ -arrestin-GFP complex is inhibited.

One way of inhibiting internalization is to prevent the binding of  $\beta$ -arrestin to clathrin and AP-2. In an improved assay according to the invention,  $\beta$ -arrestin is mutated so that its

binding to clathrin and/or AP2 is impaired.

The  $\beta$ -arrestin may be further mutated so that it, besides having impaired binding capability to clathrin and/or AP2, also is phosphorylation independent.

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As described above, the arrestins are composed of two structural and functional parts, an amino-terminal domain, which binds to the receptor and a carboxyl-terminal domain, which connects to proteins involved in receptor-sequestration, such as clathrin and AP-2. Accordingly, in one assay according to the invention, the  $\beta$ -arrestin is truncated so that it  
 10 does not contain any clathrin and/or AP2 binding sites, i.e. a part of the  $\beta$ -arrestin from the C-terminal end has been deleted.

In another assay according to the invention  $\beta$ -arrestin is mutated by deletion, insertion or substitution so that one or more AP2 binding sites of  $\beta$ -arrestin are impaired in their  
 15 binding to AP2.

Specific examples of a truncated  $\beta$ -arrestin are a human  $\beta$ -arrestin-1 374 stop mutant or human  $\beta$ -arrestin-2 373 stop mutant.

20 A specific example of a  $\beta$ -arrestin mutated by substitution is the human  $\beta$ -arrestin-2 R393E;R395E mutant, wherein the amino acids number 393 and 395 have been changed from arginine to glutamic acid. Other substitutions may of course be used.

As described above, there are at least 4 family members of arrestins. Furthermore, the  
 25 arrestins are found in animals including rodents, swine, poultry, cattle, sheep, goats, horses, cats, dogs, monkeys and humans. Thus, the specific mutations mentioned only tend as illustrative examples. All arrestins are usable in an assay according to the invention, and the specific position of the truncation and other mutations will depend on the species and type of arrestin. As an example, to impair the AP2 binding sites in bovine  
 30  $\beta$ -arrestin-2 amino acids number 394 and 396 should be substituted from arginine to glutamic acid as compared to the R393E and R395E substitutions in human  $\beta$ -arrestin.

#### **Applications of the invention**

The improved BRET assay may be used in drug discovery methods, such as screening  
 35 assays for identifying new ligands of GPCRs. The BRET assay may also be used for the discovery of ligands that interact with GPCRs of unknown function i.e. orphan GPCRs.

The ligands may be agonists or antagonists. If the ligand is a known antagonist, or if the assay is set up to screen for unknown antagonists, the improved BRET assay further comprises the addition of an agonist after adding the antagonist, or suspected antagonist ligand.

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The invention also relates to an improved assay according to the invention for use in high-throughput screening.

#### Other aspects of the invention

- 10 Other aspects of the invention appear from the appended claims. The details and particulars described above and relating to the method according to the invention apply *mutatis mutandis* to the other aspects of the invention.

#### LEGENDS TO FIGURES

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Figure 1 shows internalization of the NK1 and the  $\beta$ 2AR receptors co-expressed in cells together with WT or one of the three different  $\beta$ -arrestin mutants: human  $\beta$ -arrestin-2 R393E;R395E mutant, human  $\beta$ -arrestin-2 373 stop mutant or human  $\beta$ -arrestin-2 R169E mutant.

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Figure 2 shows the specific BRET ratio of the NK1 and the  $\beta$ 2AR receptors co-expressed with WT or one of the three different  $\beta$ -arrestin mutants: human  $\beta$ -arrestin-2 R393E;R395E mutant, human  $\beta$ -arrestin-2 373 stop mutant or human  $\beta$ -arrestin-2 R169E mutant.

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The following examples are intended to illustrate the invention without limiting it thereto.

#### EXAMPLES

##### 30 NK-1 Receptor Internalization Assays

COS-7 cells in 75 cm<sup>2</sup> flask (3x10<sup>6</sup> cells/flask) were used for transfection. NK-1/Rluc receptor (2  $\mu$ g cDNA/flask) was coexpressed together with 6  $\mu$ g GFP<sup>2</sup>/ $\beta$ -arrestin 2, 6  $\mu$ g GFP<sup>2</sup>/ $\beta$ -arrestin R169E, 6  $\mu$ g GFP<sup>2</sup>/ $\beta$ -arrestin Lys 373 stop or 6  $\mu$ g GFP<sup>2</sup>/ $\beta$ -arrestin R393E, R395E. At the end of transfection period (3 – 5 hours), cells were washed twice  
 35 with PBS, trypsinised and plated at a density of 2.5x10<sup>5</sup> cells per well in 12-well plates. After 48 hours, cells were washed once with assay medium (HEPES-modified DMEM with



0.1% BSA, pH 7.4) and incubated in assay medium for at least 1 hour before being incubated with  $^{125}\text{I}$ -labeled SP (30000 cpm/well) in 0.5 ml assay medium 10 min at 37 °C. Cells were then transferred onto ice and washed twice with ice-cold PBS. Subsequently, the extracellular receptor-associated ligand was removed by washing once with 1 ml of acid solution (50 mM acetic acid and 150 mM NaCl, pH 2.8) for 12 min. The acid wash was collected to determine the surface-bound radioactivity, and the internalized radioactivity was determined after solubilizing the cells in 0.2 M NaOH and 1% SDS (NaOH/SDS) solution. Nonspecific binding for each time point was determined under the same conditions in the presence of 1  $\mu\text{M}$  unlabeled agonist (SP). After subtraction of nonspecific binding, the internalized radioactivity was expressed as a percentage of the total binding.

Figure 1 shows the internalization of the NK1-R co-expressed in cells together with WT or one of the three different  $\beta$ -arrestin mutants. The figure illustrates that the human  $\beta$ -arrestin-2 R393E;R395E mutant and the human  $\beta$ -arrestin-2 373 stop mutant are inhibiting the internalization.

#### **$\beta_2\text{AR}$ Internalization Assays**

COS-7 cells in 75  $\text{cm}^2$  flask ( $3 \times 10^8$  cells/flask) were used for transfection.  $\beta_2\text{AR}/\text{Rluc}$  receptor (1.3  $\mu\text{g}$  cDNA/flask) was coexpressed together with 6.5  $\mu\text{g}$  GFP $^2$ / $\beta$ -arrestin 2, 6.5  $\mu\text{g}$  GFP $^2$ / $\beta$ -arrestin R169E, 6.5  $\mu\text{g}$  GFP $^2$ / $\beta$ -arrestin Lys 373 stop or 6.5  $\mu\text{g}$  GFP $^2$ / $\beta$ -arrestin R393E, R395E. Receptor internalization assay was based on protocol described by Barak and Caron J Recept Signal Transduct Res 1995 Jan-Mar;15(1-4):677-90. At the end of transfection period (3 – 5 hours), cells were washed twice with PBS, trypsinized and plated at a density of  $2.5 \times 10^5$  cells per well in 12-well plates. After 48 hours, cells were washed once with assay medium (HEPES-modified DMEM with 0.1% BSA, pH 7.4) and serum-starved in the same medium for additional 2 – 3 hours before being stimulated with 1 mM isoproterenol for 10 min at 37°C. Stimulation was stopped by washing the cells with ice-cold PBS. Cells were then subjected to [ $^{125}\text{I}$ ]-pindolol binding at 4°C for 3 h and the fraction of internalized receptors determined relative to unstimulated cells. Non-specific binding was determined under the same conditions in the presence of 1  $\mu\text{M}$  pindolol.

Figure 1 shows the internalization of the  $\beta_2\text{AR}$  co-expressed in cells together with Wt or one of the three different  $\beta$ -arrestin mutants. The figure illustrates that the human  $\beta$ -arrestin-2 R393E;R395E mutant and the human  $\beta$ -arrestin-2 373 stop mutant are inhibiting the internalization.

It is also shown that the effect is most significant for the  $\beta$ 2AR receptor as compared to the NK1 receptor.

### BRET assay

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#### *Cell preparation for BRET assay:*

1. 48 hrs after transfection remove media and wash cell 1x with PBS
2. Add 1 ml 1x trypsin (T75 flask), incubate 3-5 min at 37C
3. Add 10 mL complete media
- 10 4. Transfer cells to tube and spin down (5 min, 800 rpm)
5. Remove media, resuspend cells in 10 ml PBS and count the cells, spin down (5 min, 800 rpm)
6. Resuspend in D-PBS with 1000mg/l L-Glucose (#14287) to a density of  $2 \times 10^6$  cells/ml
- 15 7. Leave cells at room temperature for at least 30 min, to stabilize readings.

#### *Assay:*

1. Dilute DeepBlueC to 50 $\mu$ M in in D-PBS with 1000mg/l L-Glucose (#14287) (light sensitive!!)
- 20 2. Transfer 100  $\mu$ l of resuspended cells into wells in 96-well white optiplate plate
3. Add 12 $\mu$ l agonist
4. Add 10  $\mu$ l of diluted DeepBlueC/well, and read plate

#### *Antagonist:*

- 25 1. Dilute DeepBlueC to 50 $\mu$ M in in D-PBS with 1000mg/l L-Glucose (#14287) (light sensitive!!)
2. Transfer 100  $\mu$ l of resuspended cells into wells in 96-well white optiplate plate
3. Add 14 $\mu$ l antagonist ( wait 5 min.)
4. Add 14 $\mu$ l agonist
- 30 5. Add 12  $\mu$ l of diluted DeepBlueC/well, and read plate

Figure 2 shows the specific BRET ratio of the NK1 and the  $\beta$ 2AR receptors co-expressed with WT and the three different  $\beta$ -arrestin mutants: human  $\beta$ -arrestin-2 R393E;R395E mutant, human  $\beta$ -arrestin-2 373 stop mutant or human  $\beta$ -arrestin-2 R169E mutant. It is  
 35 seen that the human  $\beta$ -arrestin-2 R393E;R395E mutant and the human  $\beta$ -arrestin-2 373 stop mutant are increasing the BRET signal significantly for the  $\beta$ 2AR receptor, whereas

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the effect is less pronounced for the NK1 receptor. The observed results are expected since the NK1 receptor is a class B receptor and the  $\beta$ 2AR receptor is a class A receptor.

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**CLAIMS**

1. An improved BRET assay comprising
  - i) adding a substrate to a cell comprising GPCR-Rluc fusion protein and a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$  arrestin is mutated
  - ii) adding a ligand to obtain, if possible, a GPCR-Rluc/ $\beta$ -arrestin-GFP complex, and
  - iii) measuring a BRET signal to obtain a BRET ratio

the improvement leads to an increased BRET ratio compared with the ratios obtained by use of the same process employing a  $\beta$  arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is the wild type  $\beta$ -arrestin or employing a  $\beta$ -arrestin-GFP fusion protein, wherein the  $\beta$ -arrestin is a  $\beta$ -arrestin specifically mutated so that it acts on the receptor independent of the receptors phosphorylation state.
2. An improved assay according to claim 1, wherein separation of  $\beta$ -arrestin-GFP from GPCR-Rluc/ $\beta$ -arrestin-GFP complex is delayed and/or inhibited.
3. An improved assay according to claim 1 or 2, wherein internalization of GPCR-Rluc/ $\beta$ -arrestin-GFP complex is inhibited.
4. An improved assay according to any of the preceding claims, wherein  $\beta$ -arrestin is mutated so that its binding to clathrin and/or AP2 is impaired.
5. An improved assay according to claim 4, wherein  $\beta$ -arrestin is further mutated so that it is phosphorylation independent.
6. An improved assay according to any of the preceding claims, wherein  $\beta$ -arrestin is truncated so that it does not contain any clathrin and/or AP2 binding sites.
7. An improved assay according to any of the preceding claims, wherein  $\beta$ -arrestin is mutated by deletion, insertion or substitution so that one or more AP2 binding sites are impaired in their binding to AP2.
8. An improved assay according to any of the preceding claims wherein  $\beta$ -arrestin is originating from an animal source, such as, e.g, from rodents, swine, poultry, cattle, sheep, goats, horses, cats, dogs, monkeys and humans.
9. An improved assay according to any of the preceding claims, wherein  $\beta$ -arrestin is a  $\beta$ -arrestin-1 or  $\beta$ -arrestin-2.

10. An improved assay according to any of the preceding claims, wherein the  $\beta$ -arrestin is human  $\beta$ -arrestin-1 374 stop mutant or human  $\beta$ -arrestin-2 373 stop mutant.
- 5 11. An improved assay according to any of claims 1-9, wherein the  $\beta$ -arrestin is human  $\beta$ -arrestin-2 R393E;R395E mutant.
12. An improved assay according to any of the preceding claims for use in drug discovery methods.
- 10 13. An improved assay according to any of claims 1-11 for use in high-throughput screening.
14. Use of an improved BRET assay according to any of the preceding claims for  
15 identifying a GPCR ligand.
15. Use of an improved BRET assay according to claim 14, wherein the ligand is an agonist.
- 20 16. Use of an improved BRET assay according to claim 14, wherein the ligand is an antagonist.

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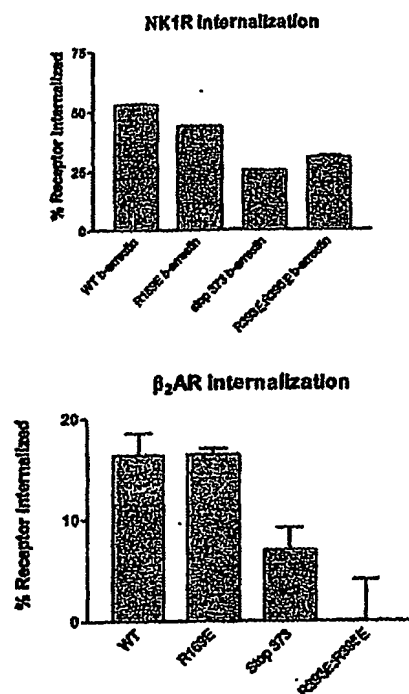


Fig 1

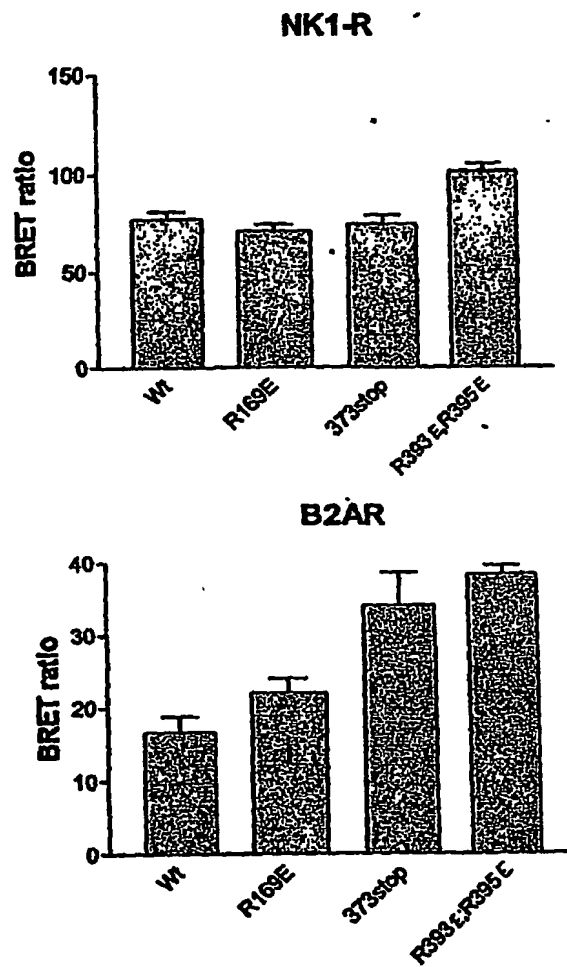


Fig 2